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[54] **VITAMIN B₁₂ MEDIATED ORAL DELIVERY SYSTEMS FOR GCSF**

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Related U.S. Application Data

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[58] **Field of Search** 530/350; 514/52; 536/26.4, 26.41, 26.42, 26.44

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[57] **ABSTRACT**

The invention describes complexes between VB₁₂ analogues and either GCSF or EPO that retain both significant affinity for intrinsic factor (IF) in the VB₁₂ portion of the complex and significant bioactivity of the GCSF or EPO portion of the complex. The invention also concerns a process for the synthesis of these complexes. This is achieved at least in part, by using a spacer compound, which is linked covalently between the VB₁₂ portion and the GCSF or EPO. The complexes preferably have the formula



wherein

V is vitamin B₁₂ or a vitamin B₁₂ analogue, or derivative, bonded to X either through a carboxylate group pendant to the corrin nucleus of VB₁₂ or through the central cobalt atom or to a functional group introduced onto the VB₁₂ molecule, X is selected from: —NHNH—, —NH—, —O—, —S—, —SS— or —CH₂—, and A is an optionally substituted, saturated or unsaturated, branched or linear, C₁₋₅₀ alkylene, cycloalkylene or aromatic group, optionally with one or more carbons within the linear chain being replaced with N, O or S, and wherein the optional substituents are selected from carbonyl, carboxy, hydroxy, amino and other groups, and Y is the covalent linkage between A and Z where Y is selected from —NHCO—, —CONH—, —CONHNHCO—, —N=N—, —N=CH—, —NHCH—, —NHN=CH—, —NHNHCH₂—, —SS—, —SCH₂—, —CH₂S—, —NHCRRNH—, —COO—, —OCO—, and R is O, S or NH₂, and Z is GCSF or EPO. The invention also describes reagents that can be used as probes for the detection of buried thiol groups of a protein or peptide, said reagent comprising a complex of either vitamin B₁₂ (or an analogue thereof) or more generally of any instrumentally or visually detectable label, covalently linked to a diradical spacer, said spacer having a terminal reactive group capable of forming a disulphide bond with a free thiol in said protein or peptides.

9 Claims, No Drawings

VITAMIN B₁₂ MEDIATED ORAL DELIVERY SYSTEMS FOR GCSF

This application is a division of application Ser. No. 08/064,873, filed May 24, 1993 now U.S. Pat. No. 5,548,064.

BACKGROUND OF THE INVENTION

The present invention relates to the oral delivery of the therapeutic substances granulocyte-colony stimulating factor (GCSF) and erythropoietin (EPO) by administration of a complex comprising these substances linked to vitamin B₁₂ (VB₁₂) or an analogue thereof. More particularly, the invention relates to methods for the synthesis of these complexes and to methods for the amplification of the amount of GCSF or EPO delivered per VB₁₂ carrier molecule.

An oral delivery system is known, because of recent work undertaken by one of the current inventors, which is described in PCT Application WO87/02251 (PCT/AU86/0299), whereby an active substance linked to at least one carrier molecule, which is VB₁₂ or an analogue thereof, can use the natural VB₁₂ uptake system mediated by the binding of VB₁₂ to intrinsic factor (IF) to transport the resultant complex from the intestinal lumen into the circulation. Once delivered into serum or the lymphatic drainage system the complex substantially retains the bioactivity of the native active substance.

In common with virtually all proteins, peptides and other large bioactive molecules there is currently no method for the oral delivery of either GCSF or EPO. The oral route of administration is the most preferable means of delivering a pharmaceutically active agent, and as such there is a large and valuable market for any process which permits the oral delivery of either of these proteins to humans. Such a process would be available by the formation of a complex between VB₁₂ and GCSF or EPO.

SUMMARY OF THE INVENTION

It is thus the object of the invention to describe complexes between VB₁₂ analogues and either GCSF or EPO that retain both significant affinity for intrinsic factor (IF) in the VB₁₂ portion of the complex and significant bioactivity of the GCSF or EPO portion of the complex. The invention also concerns a process for the synthesis of these complexes. This may be achieved at least in part, by using a spacer compound, which is linked covalently between the VB₁₂ portion and the GCSF or EPO.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

One aspect of the invention provides a complex which comprises at least one active substance linked to at least one carrier molecule, which is VB₁₂ or an analogue of VB₁₂ wherein the ability of the carrier to undergo the binding reactions necessary for uptake and transport of VB₁₂ in a vertebrate host and the activity of the active substance are substantially maintained. This occurs by providing a complex between GCSF and a carrier selected from vitamin B₁₂ or a vitamin B₁₂ analogue wherein the GCSF and the carrier are covalently linked through a diradical spacer, the complex being capable of binding to intrinsic factor with high affinity with maintenance of GCSF bioactivity.

The complex preferably has the general form V—X—A—Y—Z, where V is the carrier molecule VB₁₂ or an analogue (including derivatives) thereof that retains IF

affinity, Z is the active substance selected from EPO or GCSF, A is a spacer arm of variable composition and length, X is the functional group through which V is attached to A, and Y is the functional group through which Z is attached to A. The nature of functional group X, its site of attachment to V and the nature of spacer arm A are chosen to maximise the IF affinity of the complex. The nature of functional group Y, its site of attachment to Z and the nature of spacer arm A are chosen to maintain substantially the bioactivity of Z.

Preferably X is selected from: —NHNH—, —NH—, —O—, S—, —SS— or —CH₂—. A is preferably an optionally substituted, saturated or unsaturated, branched or linear, C₁₋₅₀ alkylene, cycloalkylene or aromatic group, optionally with one or more carbons within the linear chain being replaced with N, O or S, and wherein the optional substituents are selected from carbonyl, carboxy, hydroxy, amino and other groups. Y is preferably a covalent linkage between spacer chain A and protein Z, where Y is selected from —NHCO—, —CONH—, —CONHNHCO—, —N=N—, —N=CH—, NHCH₂, —NHN=CH—, —NHNHCH₂, —SS—, —SCH₂—, —CH₂S—, —NHCRNH— [where R=O, S or NH₂], —COO—, —OCO—, and Z is GCSF or EPO.

In the context of the present invention, the term "active substance" (ie Z) includes all, part, an analogue, homologue, derivative or combination thereof of either granulocyte colony stimulating factor (GCSF) or erythropoietin (EPO).

The carrier is VB₁₂ or VB₁₂ analogue. The VB₁₂ analogues include any variant or derivative of VB₁₂ (cyanocobalamin) which possesses binding activity to intrinsic factor. Preferred analogues of VB₁₂ also include aquocobalamin, adenosylcobalamin, methylcobalamin, hydroxycobalamin, cyanocobalamin, carbanalide, and 5-methoxybenzylcyanocobalamin [(5-MeO)CN—Cbl] as well as the desdimethyl, monocethylamide and the methylamide analogues of all of the above. Other analogues include all alkyl cobalamins in which the alkyl chain is linked to the corrin nucleus by a direct CoC covalent bond. Other analogues include chlorocobalamin, sulfocobalamin, nitrocobalamin, thiocyanatocobalamin, benzimidazolycyanocobalamin derivatives such as the: 5,6-dichlorobenzimidazole, 5-hydroxybenzimidazole, trimethylbenzimidazole, as well as adenosylcyanocobalamin [(Ade)CN—Cbl], cobalamin lactone, cobalamin lactam and the anilide, ethylamide, monocarboxylic and dicarboxylic acid derivatives of VB₁₂ or its analogues.

Preferred derivatives of VB₁₂ also include the mono-, di- and tricarboxylic acid derivatives or the propionamide derivatives of VB₁₂. Carriers may also include analogues of VB₁₂ in which the cobalt is replaced by zinc or nickel. The corrin ring of VB₁₂ or its analogues may also be substituted with any substituent which does not effect its binding to IF, and such derivatives of VB₁₂ or its analogues are part of this invention. Other derivatives of VB₁₂ or its analogues which have a functional group which is able to react with the spacer compound are also part of the invention.

It is preferred that the complex may comprise GCSF linked through a disulphide bond to a (dithiopyridyl propionamido) dodecylamino [DTP-dodecylamino] derivative of VB₁₂.

Another preferred embodiment of the invention provides a process for the production of a complex comprising GCSF linked through a disulphide bond to a (dithiopyridyl propionamido) dodecylsuberylhexylamino derivative of VB₁₂, (a long-chain analogue of the DTP-dodecylamino VB₁₂) which displays higher affinity for intrinsic factor.

Another preferred embodiment of the invention provides a process for the production of a complex comprising GCSF linked through a disulphide bond to an (dithiopyridyl propionamido) dodecylcarboxamidomethyl derivative of VB₁₂, in which the spacer is linked to the VB₁₂ through an axial CoC bond.

Another embodiment of the invention provides a process for the production of a complex comprising at least one active substance linked to at least one carrier molecule through a spacer, the carrier molecule being VB₁₂ or an analogue thereof, wherein the ability of the carrier to undergo the binding reactions necessary for uptake and transport of VB₁₂ in a vertebrate host and the activity of the active substance are substantially maintained, the process comprising one or more of the following steps:

- a) reacting together the active substance and the carrier and the spacer compound to form the complex;
- b) reacting the active substance with the spacer, and then reacting the product with the carrier to form the complex;
- c) reacting the carrier with the spacer, and then reacting the product with the active substance to form the complex;
- d) following method of (a), (b) or (c) but with the additional step of having chemically modified the carrier and/or the active substance in a previous step to provide a functional group on the carrier and/or active substance which will react with the spacer compound; or
- e) following the method of (a), (b), (c) or (d) but with the additional step of reacting the active substance or the carrier with a polymeric support, before carrying out the further reactions. Preferably the polymeric support is bonded both to the carrier (or spacer-carrier), and also to the active substance or (spacer-active substance).

For example, it is possible to form these complexes using the "e" mono -acid of "e"VB₁₂ by: (i) preparing the mono-acid derivative of VB₁₂ by mild acid hydrolysis of cyanocobalamin and purifying the initial hydrolysate; (ii) modifying the e-mono-acid to give a terminal functional group attached to the eVB₁₂ nucleus through a spacer arm; (iii) coupling the functionalised eVB₁₂ derivative to carboxylate, amine, thiol, hydroxyl, phenol, aldehyde or ketone groups or other suitable functional groups present initially or introduced chemically on the active substance. The spacer compound can be selected to have suitable functional groups at either end of its backbone, or else these functional groups can be introduced, if necessary by normal chemical synthetic reactions.

The invention also involves the modification of a polymeric support to introduce functional groups capable of reacting either directly with the spacer compound or with the spacer linked with the active substance. The resulting polymer-active substance intermediate ideally contains many molecules of the active substance, and this intermediate is suitable for coupling to the carrier to give a complex capable of amplified delivery of the active substance.

The invention also concerns a general method for the modification of unreactive thiols in peptides and proteins, particularly those not normally exposed to reagents dissolved in aqueous solvents because they are buried in hydrophobic regions of the protein. These modified peptides and proteins can then be labelled, if desired.

The invention also involves a reagent, of the general formula:

V—X—A—Y

wherein, V' is vitamin B₁₂ or a vitamin B₁₂ analogue, bonded to X either through a carboxylate group pendant to the corrin nucleus of VB₁₂ or through the central cobalt atom or to a functional group introduced onto the VB₁₂ molecule, or V' is a label, where "label" refers to any substance that is detectable by visual or instrumental methods. Labels can include chromogens, catalysts, fluorescent compounds, chemiluminescent compounds, radioactive isotopes, colloidal metal and non-metallic particles, dye particles, enzymes or substrates, antibodies or antigens, biotin, avidin or streptavidin, latex particles, liposomes or other vesicles containing signal producing substances, and the like.

X is selected from —NHCO—, —CONH—, —CONHNCO—, —N=N—, —N=CH—, —NHCH₂—, —NHN=CH—, —NHNHCH₂—, —SS—, —SCH₂—, —CH₂S—, —NHCRNH—, [R is O, S or NH₂], —COO—, —OCO—, and A is an optionally substituted, saturated or unsaturated, branched or linear, C₁₋₅₀ alkylene, cycloalkylene or aromatic group, optionally with one or more carbons within the chain being replaced with N, O or S, and wherein the optional substituents are selected from carbonyl, carboxy, hydroxy, amino and other groups, and Y' is a functional group capable of reacting with thiols to give a stable covalent linkage, including iodoacetyl, bromoacetyl, chloroacetyl, maleimido, 3-carboxy-4-nitrophenyldithio or 2-pyridyldithio, and similar groups.

BEST METHOD OF CARRYING OUT THE INVENTION

EXAMPLES

Materials: VB₁₂ was obtained from Rousell-Uclaf. GCSF and EPO were obtained from Amgen. 1-Ethyl-3-(dimethylaminopropyl) carbodiimide HCl (EDAC HCl) was obtained from Biorad. N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and succinimidyl, 6-[3-(2-pyridyldithio) propionamido] hexanoate (LC—SPDP) were obtained from Pierce Chemical Co. All other reagents were obtained from Fluka.

Example 1

VB₁₂ -GCSF complexes

Three classes of VB₁₂-GCSF complexes were prepared:

- a) conjugated via an amide linkage, formed by carbodiimide (EDAC) mediated coupling of an amino terminal eVB₁₂ derivative to the C-terminus of GCSF or the carboxylate side chains of GCSF.
- b) conjugated via a disulphide linkage formed by a thiol insertion reaction of the free thiol at Cys-17 of GCSF into the disulphide bond present in the (dithiopyridylpropionamido) terminal derivatives of eVB₁₂.
- c) conjugated via an acyl hydrazide linkage, formed by EDAC-mediated coupling of a hydrazido-terminal eVB₁₂ derivative to the C-terminus of GCSF or the carboxylate side chains of GCSF.

1.1 Production and purification of the "e" isomer of monocarboxy-VB₁₂.

The "e" isomer of monocarboxy vitamin B₁₂, formerly named the d isomer, but reassigned by Anton and co-workers (1980: J. Am. Chem. Soc. 102:2215) as the e isomer, was separated from the b and d isomers formed during acid hydrolysis of cyanocobalamin by a combination of Dowex 1X2 chromatography and semi-preparative C-18 RP-HPLC developed with a gradient of acetonitrile in 0.1% TFA.

1.2 Production of amino-derivatives of eVB₁₂

A number of amino-derivatives of eVB₁₂ were prepared by reacting the e isomer with:

- i) 1,2-diaminoethane
- ii) 1,6-diaminohexane
- iii) 1,12-diaminododecane
- iv) 1,3-diamino-2-hydroxypropane
- v) 1,6-diamino-3,4-dithiahexane (a.k.a cystamine)

All reactions were performed at pH 6.5 using a twenty fold molar excess of the diamine over e isomer and a twenty fold molar excess of EDAC. In a typical reaction 135 mg of eVB₁₂ was dissolved in distilled water (6 ml) to which was added 1.2 ml of 1.0M diamine, pH 6.5. Dry EDAC (270 mg) was then added and the reaction mixture was left overnight at room temperature.

All amino derivatives were purified by reverse phase chromatography on a semi-preparative C-4 column using a 5-100% acetonitrile gradient in 0.1% TFA. Eluted material was further purified by S-Sepharose chromatography. The amino-derivative was eluted with 0.1M HCl, followed by extraction into phenol, and back-extraction into water after the addition of dichloromethane to the phenol phase. The amino-eVB₁₂ derivatives were then recovered from the water phase by lyophilization.

1.3 Conjugation of 2-aminoethyl-eVB₁₂ to GCSF

A solution of 2-aminoethyl-eVB₁₂ (26.5 mg, 18 μ mol) in 2 ml of GCSF (6 mg/ml, 0.63 μ mol) was cooled to 4 C. An aliquot of freshly prepared EDAC solution (100 mg/ml, 120 μ l, 63 μ mol) was added. After 24 h at 4 C a second aliquot of freshly prepared EDAC solution was added. The reaction was allowed to proceed for a total of 48 h at 4 C, after which the unreacted 2-aminoethyl-eVB₁₂ was separated from the conjugate and aggregate by chromatography on Sephadex G-50 in 2.5% acetic acid.

1.4 Conjugation of cystamyl-eVB₁₂ to GCSF

A solution of cystamyl-eVB₁₂ hydrochloride (30 mg, 20 μ mol) in 2.5 ml of GCSF (6 mg/ml, 0.80 μ mol) at 4 C was treated with an aliquot of an aqueous EDAC solution (20 mg/ml, 75 μ l, 8 μ mol). Further aliquots of freshly prepared EDAC solution (20 mg/ml, 75 μ l, 8 μ mol) were added after 4.5 h, 7 h and 24 h. The reaction was allowed to proceed for a total of 48 h at 48 C, after which time the unreacted amino-eVB₁₂ was separated from the conjugate by chromatography on Sephadex G-50 in 2.5% acetic acid.

1.5 Preparation of 3-(2-pyridylthio) propionamido derivatives of aminoethyl-eVB₁₂

The thiopyridyl derivatives of amino-eVB₁₂ were prepared by reacting SPDP with:

- i) 2-aminoethyl-eVB₁₂
- ii) 6-aminoethyl-eVB₁₂
- iii) 12-aminododecyl-eVB₁₂

In a typical reaction the terminal amino-eVB₁₂ was dissolved at 50 mg/ml in 0.1M PO₄ buffer, pH 7.5, containing 0.1M NaCl. SPDP was dissolved at 50 mg/ml in acetone and 800 μ l of the solution was added to the amino-eVB₁₂. After reaction overnight at room temperature the DTP-amino-eVB₁₂ was purified by RP-HPLC on a semi-prep C4 column, and then lyophilized.

1.6 Conjugation of DTP-amino-VB₁₂ to GCSF

In initial conversations with AMGEN it was revealed that they had found that it was impossible to modify the free cysteine in undenatured GCSF with standard thiol modifying agents. Initial experiments with DTP-aminoethyl-eVB₁₂ showed that it was possible to achieve some 20% substitution of GCSF with the VB₁₂ in the absence of guanidine; this level rose to >80% in the presence of 4M guanidine. It was

therefore decided that it might be possible to access the free thiol with DTP-amino-eVB₁₂ in the absence of guanidine if a longer spacer was used for the conjugation.

In a second series of experiments GCSF was reacted with DTP-aminoethyl-, DTP-aminoethyl- and DTP-aminododecyl-eVB₁₂ in the presence or absence of 4M guanidine in 0.1M sodium acetate buffer, pH 4.0.

The degree of substitution of GCSF by various DTP-amino-eVB₁₂-spacer complexes is shown in the following table:

TABLE 1

Spacer	Guanidine	+ Guanidine
DTP-aminoethyl-	37.5%	89.3%
DTP-aminoethyl-	45.5%	95.2%
DTP-aminododecyl-	100.0%	100.0%

From Table 1 it can be seen that by switching to the longer dodecyl-spacer it was possible to conjugate to the buried thiol in GCSF without the use of guanidine.

The initial attempts to conjugate to the free thiol group in GCSF using the pyridylthiopropionamido-aminoethyl derivative of eVB₁₂ resulted in a small degree of conjugation, of around 20-40 percent in the absence of guanidine. The addition of 4M guanidine (final concentration) raised the conjugation efficiency to over 80%. Preparation of a longer, more hydrophobic derivative of eVB₁₂, pyridylthiopropionamido-dodecyl eVB₁₂ resulted in 100% substitution of GCSF after 24 h at 4 C, without the need for the addition of guanidine. The use of the thiol interchange chemistry in this reaction proved advantageous as the eVB₁₂ conjugation was surprisingly successful at pH's which minimised the extent to which GCSF undergoes spontaneous aggregation. Chromatography of the conjugated material resulted in base-line separation of conjugate from free eVB₁₂.

This type of approach can be more generally applied for the development of reagents capable of detecting, quantifying and/or modifying thiol groups in proteins and peptides which were hitherto regarded as chemically unreactive.

1.7 Scale up conjugation of DTP-aminododecyl-eVB₁₂ to GCSF

Following the initial success with the conjugation of the DTP-dodecyl-spacer, the reaction was scaled up as follows:

To 2.5 ml of GCSF (6 mg/ml; 15 mg) was added 1.6 ml of DTP-aminododecyl-eVB₁₂ (10 mg/ml in 2.5% acetic acid). The reaction was allowed to proceed for 48 h at 4 C, after which the unreacted eVB₁₂ was separated from the conjugate by chromatography on Sephadex G-25 in 2.5% acetic acid. Fractions containing GCSF were pooled, concentrated in an AMICON positive pressure cell using a YM 10 membrane and dialysed for 72 h against sterile distilled water.

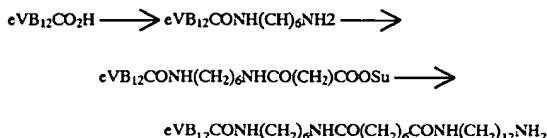
1.8 Preparation of DTP-dodecylsulberhexyl-eVB₁₂ reagent.

Although the DTP-dodecyl-eVB₁₂ reagent reacts efficiently with GCSF to give a stable, well characterised complex, the material had a low IF affinity (~2-3% of native eVB₁₂). A complex with increased IF affinity was prepared by synthesising an extended spacer analogue of the DTP-dodecylamino-eVB₁₂. The synthesis of this analogue uses the same SPDP chemistry to conjugate through the cysteine of GCSF; however a longer spacer arm is attached to the eVB₁₂. As anticipated, this resulted in the formation of a conjugate whose IF affinity is significantly greater than that of the conjugate prepared by the reaction of GCSF with DTP-dodecyl-eVB₁₂ (see Table 2).

This spacer was prepared by sequential reaction of the e-carboxylate of eVB₁₂ with:

- i) 1,6-diaminohexane and EDAC (to give 6-aminohexyl eVB₁₂).
- ii) disuccinimidyl suberate (DSS) (to give monosuccinimidylsuberylhexyl-eVB₁₂).
- iii) 1,12-diaminododecane (to give 12-aminododecylsuberylhexyl-eVB₁₂).

That is:



The resultant spacer, which is more than twice the length of aminododecyl-eVB₁₂, was derivatized at the terminal amino group with SPDP and coupled to GCSF by means of the protocol described in the following section.

1.9 Conjugation of DTP-dodecylsuberylhexyl-eVB₁₂ to GCSF

A solution of the DTP-dodecylsuberylhexyl-eVB₁₂ (9 mg, 7 μmol) was taken up in acetic acid (100 μl) and diluted to 1 ml with water. This solution was added to 5 ml of GCSF solution (4 mg/ml, 0.5 μmol) cooled to 4 C. The reaction mixture was left for 144 h at 4 C, then worked up using the standard protocol.

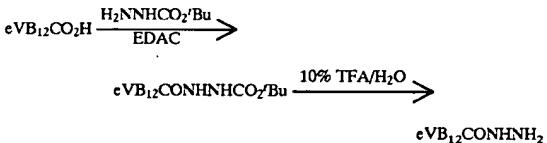
1.10 Production of hydrazide derivatives of eVB₁₂ carboxylate

Two hydrazide derivatives of eVB₁₂ carboxylate were prepared for conjugation to carboxyl groups of GCSF by reaction with EDAC. The two hydrazide derivatives used, and their (shorthand) chemical structure, are:

- a) hydrazido-eVB₁₂ (=eVB₁₂—CONHNH₂)
- b) adipyl-hydrazido-eVB₁₂ (=eVB₁₂—CONHNHCO(CH₂)₄CONH₂)

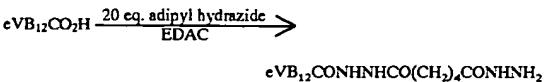
1.10i Hydrazido-eVB₁₂

This reagent was prepared by a two step synthesis involving the coupling of tert butyl carbazate to eVB₁₂ carboxylate and subsequent removal of the tBoc group to generate the free hydrazide.



1.10ii Adipyl-hydrazido-eVB₁₂

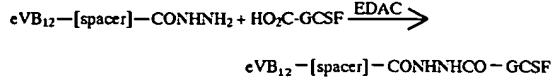
This reagent was readily prepared in one step from eVB₁₂ carboxylate by the addition of EDAC to a mixture of the acid and a 20-fold excess of adipylhydrazide.



EDAC-mediated coupling of hydrazido-eVB₁₂ analogues to the carboxylate side-chains of GCSF proceeded more readily, and required significantly lower amounts of eVB₁₂ derivative and EDAC, than the corresponding conjugations of amino eVB₁₂ derivatives to GCSF. This is readily explainable in terms of the relative basicity of hydrazides (pKa 2.6) in comparison with amines (pKa 8–9). Thus at the pH at which the GCSF coupling takes place (4–5) a

hydrazido eVB₁₂ derivative will be primarily in the reactive, non-protonated form while an amino-eVB₁₂ derivative will be primarily in the non-reactive, protonated form.

The coupling reaction used is:



1.11 Conjugation of hydrazido-eVB₁₂ to GCSF

A solution of hydrazido eVB₁₂ (8.9 mg, 6.5 μmol) in 5 ml of GCSF solution (4 mg/ml, 1.05 μmol) was cooled to 4 C and an aliquot of EDAC solution (50 mg/ml, 40 μl , 10 μmol) was added. After 5 h an identical aliquot of fresh EDAC solution was added and the reaction mixture was left overnight at 4 C. Conjugate was removed from unreacted eVB₁₂ and other reagents by chromatography on Sephadex G-50 in 2.5% acetic acid.

1.12 Conjugation of adipylhydrazido-eVB₁₂ to GCSF

A solution of adipylhydrazido eVB₁₂ (10 mg, 6.6 μmol) in 3 ml of GCSF solution (4 mg/ml, 0.64 μmol) was cooled to 4 C and an aliquot of EDAC solution (50 mg/ml, 40 μl , 10 μmol) was added. After 5 h a 20 μl aliquot of fresh EDAC solution (50 mg/ml, 20 μl , 5 μmol) was added and the reaction mixture was left overnight at 4 C. Conjugate was removed from unreacted eVB₁₂ and other reagents by chromatography on Sephadex G-50 in 2.5% acetic acid.

1.13 Preparation of poly-GCSF-HPMA—eVB₁₂ complex.

Two N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers can be synthesized as polymer backbones for the incorporation and derivatization with GCSF and eVB₁₂. A non-biodegradable polymer backbone (HPMA-GG) can be synthesized by the free radical copolymerization of HPMA with N-methacryloylglycylglycine p-nitrophenyl ester. A biodegradable polymer (HPMA-FALG) can be synthesized by the free radical copolymerization of HPMA with N-methacryloylglycylphenylalanyleucylglycine p-nitrophenol ester by the method of Rejmanova and coworkers [Rejmanova, P, Obereigner, B and Kopecek, J, 1981 *Makromol Chem* 182:1899–1915]. In order to incorporate GCSF and eVB₁₂ onto the polymers, the polymers are initially reacted with a ten molar excess of a mixture of aminododecyl-eVB₁₂ and dithiopyridyldecylsuberylhexylamine (1:10 mole:mole) overnight. Unreacted nitrophenyl esters are subjected to aminolysis by the addition of 1-amino-2-propanol. The modified polymers are purified by chromatography on Sepharose 6B. A solution of the dithiopyridyldecylsuberylhexyl modified eVB₁₂-substituted polymers is dissolved in 2.5% acetic acid and reacted with GCSF. The reaction mixture is left for 144 h at 4 C, after which the GCSF-eVB₁₂-substituted polymers can be purified by chromatography on Sepharose 6B.

Part 2: eVB₁₂-EPO complexes

Three classes of eVB₁₂-EPO complexes were prepared:

- (a) Conjugated via an amide linkage, formed by EDAC-mediated coupling of an amino eVB₁₂ derivative to the C-terminus of EPO, the carboxylate side chains of the Asp/Glu residues of EPO or the carboxylate groups of the sialic acid residues of the carbohydrate portion of EPO.
- (b) Conjugated via an acyl hydrazide linkage, formed by EDAC-mediated coupling of a hydrazido-eVB₁₂ derivative to the carboxylate side chains of the Asp/Glu residues of EPO or the carboxylate groups of the sialic acid residues of the carbohydrate portion of EPO.
- (c) Conjugated via a hydrazone linkage between a hydrazido-eVB₁₂ derivative and an aldehyde group

generated by periodate oxidation of the carbohydrate residues of EPO.

2.1 Conjugation of 2-aminoethyl- ϵ VB₁₂ to EPO

A mixture of 2-aminoethyl- ϵ VB₁₂ (8 mg, 5.7 μ mol) and EPO (27 mg/ml, 200 μ l, 0.18 μ mol) was cooled to 4 C and an aliquot of EDAC solution (10 mg/ml, 100 μ l, 5 μ mol) was added. The reaction mixture was left for 64 h at 4 C and finally purified by size-exclusion chromatography on a Superdex-75 column. Elution with a buffer consisting of Tris (pH 7.5, 10 mM; NaCl, 100 mM) afforded the purified EPO- ϵ VB₁₂ complex.

2.2 Conjugation of cystaminyl- ϵ VB₁₂ to EPO

A mixture of 6-amino-3,4-dithiahexylamide- ϵ VB₁₂ (12 mg, 8.1 μ mol) and EPO (13.5 mg/ml, 500 μ l, 0.23 μ mol) was cooled to 4 C and an aliquot of EDAC solution (10 mg/ml, 250 μ l, 13 μ mol) was added. The reaction mixture was left for 48 h at 4 C and was finally purified by size-exclusion chromatography on a Sephadex G-75 column. Elution with a buffer consisting of Tris (pH 7.5, 10 mM)/NaCl (100 mM) afforded the purified EPO- ϵ VB₁₂ complex.

2.3 EDAC-mediated conjugation of hydrazido- ϵ VB₁₂ to EPO

A solution of hydrazido VB₁₂ (10 mg, 7.3 μ mol) in 7 ml of EPO solution (2.6 mg/ml, 0.6 μ mol) was cooled to 4 C and an aliquot of EDAC solution (20 mg/ml, 50 μ l, 5 μ mol) was added. After 5 h a second aliquot of fresh EDAC solution (10 mg/ml, 25 μ l, 1.3 μ mol) was added and the reaction mixture was left overnight at 4 C. The conjugate was purified by size-exclusion chromatography on a G-50 column. Elution with a buffer consisting of Tris (pH 7.5, 10 mM; NaCl, 100 mM) afforded the purified EPO- ϵ VB₁₂ complex.

2.4 EDAC-mediated conjugation of adipyl-hydrazido- ϵ VB₁₂ to EPO

A solution of adipyl-hydrazido- ϵ VB₁₂ (11 mg, 7.3 μ mol) in 4 ml of EPO solution (2.6 mg/ml, 0.35 μ mol) was cooled to 4 C and an aliquot of EDAC solution (10 mg/ml, 100 μ l, 5 μ mol) was added. The reaction mixture was left overnight at 4 C. The conjugate was purified by size-exclusion chromatography on a G-50 column. Elution with a buffer consisting of Tris (pH 7.5, 10 mM)/NaCl (100 mM) afforded the purified EPO- ϵ VB₁₂ complex.

2.5 Periodate mediated conjugation of hydrazido- ϵ VB₁₂ to EPO

An aqueous solution of EPO (6.4 mg/ml, 1.56 ml, 0.33 μ mol) was cooled to 4 C and freshly prepared sodium periodate solution (25 mM, 360 μ l) was added. The solution was left to stir gently for fifteen minutes at 4 C and the excess periodate was quenched by the addition of ethylene glycol (5 μ l). The solution was dialyzed overnight against 3

1 of pH 5.6, 10 mM NaOAc buffer. A solution of hydrazido- ϵ VB₁₂ (7.5 mg, 5 μ mol) in distilled water (200 μ l) was added to the oxidised EPO solution and the reaction mixture was left for 7 h at 4 C. The conjugate was separated from unreacted hydrazide by SEC on G-75 Sephadex. Elution with a buffer consisting of Tris (pH 7.5/10 mM)/NaCl (100 mM) afforded the purified EPO- ϵ VB₁₂ complex.

2.6 Preparation of poly-EPO-HPMA- ϵ VB₁₂

Two N-(2-hydroxypropyl)methacrylamide (HPMA) 10 copolymers can be synthesized as polymer backbones for the incorporation and derivatization with EPO and VB₁₂. A nonbiodegradable polymer backbone (HPMA-GG) is synthesized by the free radical copolymerization of HPMA with N-methacryloylglycylglycine p-nitrophenyl ester. A biodegradable polymer (HPMA-GFLAG) is synthesized by the free radical copolymerization of HPMA with N-methacryloylglycyl-phenylalanylleucylglycine p-nitrophenol ester by the method of Rejmanova and coworkers [Rejmanova, P, Obereigner, B and Kopecek J, 1981 *Makromol Chem* 182:1899-1915]. In order to incorporate EPO and ϵ VB₁₂ onto the polymers, the polymers are reacted with a ten molar excess of a mixture of aminododecyl- ϵ VB₁₂ and 6-(3-acylhydrazidylpropionamido)-1-amino-3,4-dithiahexane (1:10mole:mole)overnight. Unreacted nitrophenyl esters are subjected to aminolysis by the addition of 1-amino-2-propanol. The modified polymers are purified by chromatography on Sepharose 6B. EPO is covalently linked to the 6-(3-acylhydrazidylpropionamido)-1-amino-3,4-dithiahexane modified ϵ VB₁₂-substituted polymers by the addition of EDAC. The reaction mixture is left for 18 h at 4 C, after which the EPO- ϵ VB₁₂-substituted polymers are purified by chromatography on Sepharose 6B.

Example 3

The diradical spacer may be selected with a suitable length and functionality to optimize the intrinsic factor (IF) affinity of the resulting GCSF or EPO ϵ VB₁₂ complex. As examples of this, the following table shows the result of 40 altering the length and functionality of the spacer for various complexes and VB₁₂ derivatives.

The IF affinity of the various ϵ VB₁₂ analogues and complexes referred to in the Examples are shown in Table 2. In particular, there is significant increase in IF affinity 45 between the GCSF-dithiopropionamido[12-aminododecylamido]- ϵ VB₁₂ (prepared in section 1.7) and the GCSF-dithiopropionamido[12-aminododecyl-suberylhexylamido]- ϵ VB₁₂ (prepared in section 1.9). This increase in IF affinity presumably results from the increase in length of the spacer arm in the latter complex.

TABLE 2

Relative Intrinsic Factor affinity of VB ₁₂ analogues and complexes	
ϵ VB ₁₂ Analogue or complex	IF Affinity
ϵ VB ₁₂ carboxylate	35%
2-aminoethylamido- ϵ VB ₁₂	48%
6-aminohexylamido- ϵ VB ₁₂	91%
12-aminododecylamido- ϵ VB ₁₂	74%
12-aminododecyl-suberyl-hexylamido- ϵ VB ₁₂	82%
dithiopropionamido[2-aminoethylamido]- ϵ VB ₁₂	6%
dithiopropionamido[6-aminohexylamido]- ϵ VB ₁₂	7%
dithiopropionamido[6-aminohexylamido]- ϵ VB ₁₂	11%
GCSF-dithiopropionamido[12-aminododecylamido]- ϵ VB ₁₂	3%
GCSF-dithiopropionamido[12-aminododecyl-suberyl-hexylamido]- ϵ VB ₁₂	28%
hydrazidyl- ϵ VB ₁₂	100%

TABLE 2-continued

Relative Intrinsic Factor affinity of VB ₁₂ analogues and complexes	
cVB ₁₂ Analogue or complex	IF Affinity
adipyldihydrazidyl-eVB ₁₂	44%
GCSF-acyldihydrazidyl-eVB ₁₂	10%
GCSF-acyl[adipyldihydrazidyl]-eVB ₁₂	7%
EPO-acyldihydrazidyl-eVB ₁₂	6%
EPO-acyl[adipyldihydrazidyl]-eVB ₁₂	11%

IF Affinity is expressed as a percentage of the affinity of the eVB₁₂ derivative relative to native, unmodified VB₁₂.

We claim:

1. A covalent conjugate comprising GCSF and vitamin B₁₂ or an analogue of vitamin B₁₂ said GCSF being covalently bound to said vitamin B₁₂ or analogue of vitamin B₁₂ by a diradical spacer such that said conjugate binds to intrinsic factor with high affinity and maintains GCSF bioactivity.

2. A complex according to claim 1 wherein said diradical spacer is covalently linked to GCSF through: a disulphide bond with Cys-17 of GCSF; an amide linkage; an acyl hydrazone linkage; an imine linkage; or a hydrazone linkage.

3. A covalent conjugate according to claim 1 wherein said vitamin B₁₂ analogue is an analogue where the corrin ring is substituted with any of a group of corrin ring constituents selected from the group consisting of: cyanocobalamin, aquocobalamin, adenosylcobalamin, methylcobalamin, hydroxocobalamin, and their desdimethyl, monoethylamide, and methylamide derivatives; alkyl cobalamins in which the alkyl chain is linked to the corrin nucleus by a direct cobalt-carbon covalent bond; chlorocobalamin, sulfitocobalamin, nitrocobalamin, thiocyanatocobalamin; the anilide, ethylamide, propionamide, monocarboxylic and dicarboxylic acid derivatives of vitamin B₁₂, and its analogues; the monocarboxylic, dicarboxylic, and tricarboxylic acid derivatives and the propionamide derivatives of the "e" isomer of monocarboxy vitamin B₁₂; and analogues of vitamin B₁₂ in which the cobalt is replaced by zinc or nickel.

4. A covalent conjugate according to claim 1 wherein said GCSF is covalently bound to a pharmaceutically acceptable polymer.

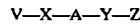
5. A covalent conjugate according to claim 4 wherein the polymer is selected from dextran, inulin, cellulose, starch and derivatives thereof, chondroitan sulfate, poly[N- α -(2-hydroxypropyl)-methacrylamide] and derivatives thereof, styrene-maleic anhydride copolymer, divinylether-maleic anhydride copolymer, polylysine, poly(glutamic acid), poly(hydroxypropyl glutamine), poly(lactic acid), water soluble polyurethanes formed by covalent linkage of PEG with lysine or other amino acids and branched chain polypeptides.

6. A covalent conjugate according to claim 4 wherein said pharmaceutically acceptable polymer is biodegradable within the human or animal body.

15 7. A covalent conjugate according to claim 4, wherein the polymer is selected from dextran, inulin, cellulose, starch and derivatives thereof, chondroitan sulfate, poly[N- α -(2-hydroxy-propyl)-methacrylamide] and derivatives thereof, styrene-maleic anhydride copolymer, divinylether-maleic anhydride copolymer, polylysine, poly(glutamic acid), poly(hydroxypropyl glutamine), poly(lactic acid), water soluble polyurethanes formed by covalent linkage of PEG with lysine or other amino acids and branched chain polypeptides.

20 8. A pharmaceutical composition which comprises a covalent conjugate according to claim 1 and a pharmaceutically acceptable carrier or excipient.

25 9. A covalent conjugate according to claim 1 of the formula:



wherein,

35 V is vitamin B₁₂ or an analogue of vitamin B₁₂ or derivative, bonded to X either through a carboxylate group pendant to the corrin nucleus of vitamin B₁₂ or through the central cobalt atom or to a functional group introduced onto the vitamin B₁₂ molecule,

40 X is selected from: —NHNH—, —NH—, —O—, S—, —SS— and —CH₂—, and A is an optionally substituted, saturated or unsaturated, branched or linear, C₁₋₅₀ alkyne, cycloalkyne or aromatic group, optionally with one or more carbons within the linear chain being replaced with N, O or S, and wherein the optional substituents are selected from carbonyl, carboxy, hydroxy, amino and other groups, and

45 Y is the covalent linkage between A and Z where Y is selected from —NHCO—, —CONH—, —CONHNHCO—, —N=N—, —N=CH—, —NHCH₂—, —NHN=CH—, —NHNHCH₂—, —SS—, —SCH₂—, —CH₂S—, —NHCRNH—, —COO—, —OCO—, wherein R is O, S or NH₂, and

50 Z is GCSF.

* * * * *